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Report on the preparation of deuterium-labelled aconitine and mesaconitine and their application to the analysis of these alkaloids from body fluids as internal standard

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Abstract

Improved analysis of aconitine and mesaconitine, highly toxic compounds from *Aconitum* species, in body fluids by gas chromatography–selected ion monitoring with their deuterium-labelled analogues as internal standards (I.S.s) is described. Deuterium-labelled analogues of aconitine and mesaconitine were synthesized by the substitution of the *N*-alkyl group for a deuterium-labelled one. The mass spectra of the derivatives of the deuterium labels closely resembled that of the nonlabelled compounds except for an obvious mass shift produced by substitution of the deuterium atoms at *N*-alkyl groups. Using these deuterium-labelled compounds as I.S.s, the standard curves for aconitine and mesaconitine were linear (r^2=0.999 each) in the concentration range of 50 pg to 50 ng, respectively. The detection limit of the alkaloids was 10 pg each per injection. The recovery, accuracy and precision of the analysis were evaluated with three different concentration of spiked human blood and urine (n=5 each). The recovery rates ranged from 97.6% to 101.3% and the standard deviations of the interseries ranged from 2.1% to 3.9%. These I.S.s give us a more precise analysis and may be useful in examining the behavior of these alkaloids in the human body. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Aconitine; Mesaconitine

1. Introduction

Aconitum plants (Ranunculaceae) are widely distributed across northern Asia and North America and contain highly toxic *Aconitum* alkaloids. Aconitine and mesaconitine are the major toxic *Aconitum* alkaloids. The lethal dose 50% (LD_{50}) of aconitine for mice is 1.8 mg/kg (orally) and 0.308 mg/kg (intraperitoneally) [1] and the lethal dose of these alkaloids for humans is estimated to be 1 to 2 mg [2]. Poisoning by *Aconitum* alkaloids has been famous since ancient times. Thus, aconites have been used for over 2000 years in Eurasia as a homicidal agent, sometimes in the form of an arrow poison. Even today, aconites are sometimes used as homicidal or suicidal agents. From the viewpoint of forensic medicine, identification and determination of the toxicant from biological fluids is necessary to specify the causes of poisoning.

In contrast, tubers of *Aconitum* plants after detoxication to a considerable extent with heat or by chemical treatments, are one of the most important ingredients of Chinese medical preparations due to their pharmacological effects, i.e., antiinflammatory

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effects [3] and antipain effects [4]. Studies on alkaloids have revealed that the essence of the pharmacological effects of aconite are attributed to diester type *Aconitum* alkaloids, including aconitine and mesaconitine [3,4]. Although they have these attractive pharmacological effects, there have been many reports of aconite poisoning during clinical use [5–7]. Therefore, more efficacious treatment using aconites is possible by therapeutic drug monitoring.

Since a low dose of diester type *Aconitum* alkaloids can cause poisoning, the concentration of the alkaloids in body fluids is very low. Although several methods have been reported concerning the quantitative determination of *Aconitum* alkaloids, such as thin-layer chromatography (TLC) [8], paper partition chromatography [9], gas (GC)–liquid chromatography [10] and high-performance liquid chromatography [11], these methods have drawbacks such as incomplete resolution and imprecise determination. On the other hand, GC–selected ion monitoring (GC–SIM) gives us a specific and precise analysis and has great advantages for the analysis of biological fluids [12]. In a previous paper, we described the analysis of *Aconitum* alkaloids from the tubers of *Aconitum japonicum* Thunb. [13], and from human serum by GC–SIM [14].

Choosing stable isotopes as suitable internal standards (I.S.s) is important in trace analysis to compensate for loss of reactivity caused during the sample preparation. Thus, preparation of stable isotope-labelled I.S.s for GC–SIM analysis is desirable for a more precise analysis.

In this present study, we report on the preparation of deuterium-labelled aconitine and mesaconitine and their application to the analysis of these alkaloids from body fluids as I.S.s.

2. Experimental

2.1. Reagents

Aconitine was purchased from Sigma (St. Louis, MO, USA), mesaconitine from Kishida (Osaka, Japan), osmium tetraoxide from Wako (Osaka, Japan) and BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide) from Tokyo Kasei Kogyo (Tokyo, Japan). d_5-Iodoethane and d_3-iodomethane were purchased from Aldrich (Milwaukee, WI, USA). Solvents and all other reagents were of analytical grade.

2.2. Preparation of deuterium-labelled aconitine

An outline of the preparation of deuterium-labelled aconitine and mesaconitine is shown in Fig. 1. The principles of preparation of deuterium-labelled aconitine are: (1) aconitine is converted into *N*-desethylaconitine by oxidation with OsO_4 according to the method of Desai et al. [15], and (2) *N*-desethylaconitine is reacted with d_5-iodoethane and converted to d_5-aconitine.

Briefly, 50 mg of aconitine was dissolved in 2 ml of pyridine and then reacted with 50 mg of OsO_4 in 2 ml of dioxane. The mixture was stirred at room temperature for 3 h until the aconitine disappeared after sampling by TLC. $NaSO_3$ (100 mg) in 5 ml of H_2O was poured into the reaction mixture and stirred for 1 h to end the reaction. The reaction mixture was then extracted three times using 30 ml of CH_2Cl_2 each time. The organic layer was collected and washed twice with 90 ml of H_2O, dried with anhydrous $NaSO_4$ and then evaporated in vacuo. The residue was dissolved in 50 ml of $CHCl_3$ and the solution was extracted three times with 3 ml of 1.5% H_2SO_4. The aqueous layer was combined and washed twice with 10 ml of $CHCl_3$, and that basified with 1 *M* NaOH and extracted three times with 30 ml of $CHCl_3$. The organic phase was collected, evaporated and purified by alumina (neutral, grade 3) column chromatography.

N-Desethylaconitine (20 mg) was dissolved with 10 ml of ether and methanol (1:1), and about 100 mg of $CaCO_3$ was added. The mixture was sealed and reacted with 50 μl of d_5-iodoethane at 60°C for 1 h. The deuterium-labelled analogue of aconitine was extracted with $CHCl_3$ (30 ml×3) and the solvent was evaporated. The analogue was purified by alumina (neutral, grade 3) chromatography and recrystallized from ether–hexane. For the preparation of deuterium-labelled mesaconitine, d_3-iodomethane was used instead of d_5-iodoethane.

2.3. GC–mass spectrometry (GC–MS)

A DX303 GC–MS system (Jeol, Tokyo, Japan) with an all-glass Vandenberg-type solventless injector interfaced with a DA-5000 data processing system

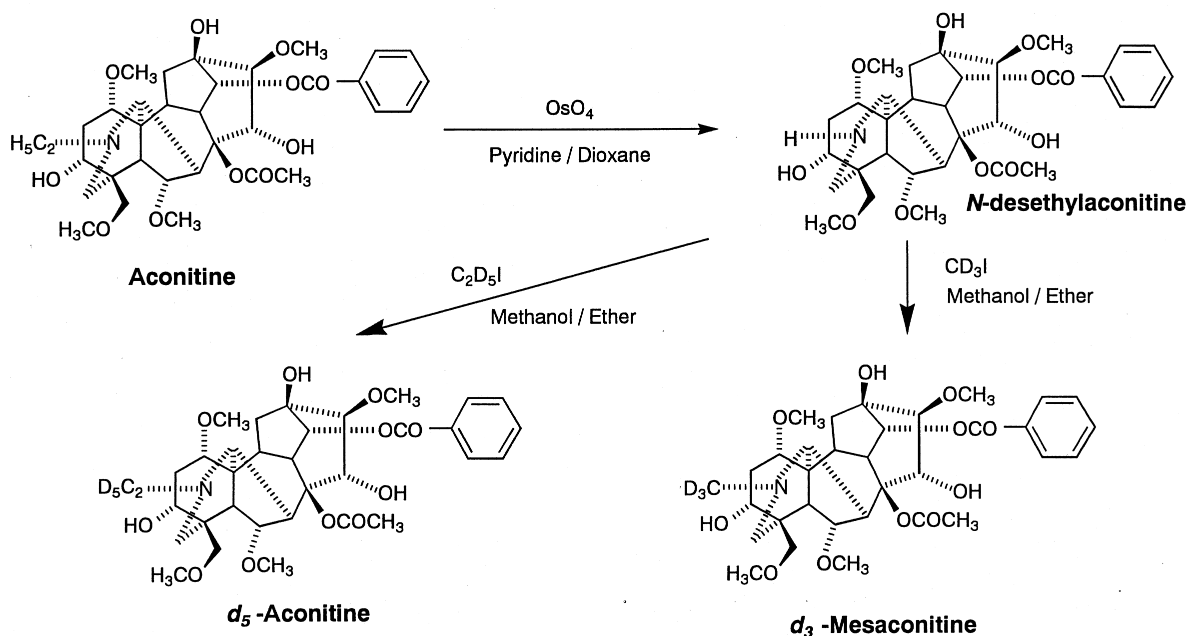

Fig. 1. Reaction conditions for the synthesis of d_5-aconitine and d_3-mesaconitine.

(Jeol) was employed. The column was a 15 m×0.25 mm I.D. fused-silica capillary cross-linked column with methyl 5% phenylsilicone (DB-5, J&W Scientific, Folsom, CA, USA). The temperature of the column oven was maintained at 250°C for 1 min, then programmed to 320°C at 16°C/min. The carrier gas was helium, with a linear velocity of 25 cm/s. The temperature of the injection port and transfer line was kept at 320°C and that of the ion source at 250°C. The ionization energy and trap current were 70 eV and 300 μA, respectively. The accelerating voltage was 3 kV. The mass spectra of each trimethylsilyl (TMS) derivative of aconitine, mesaconitine and their deuterium-labelled analogues were recorded by scanning over a mass range at *m/z* 100–900 (scanning time 2 s) with a dynamic resolution of 1000.

2.4. GC–SIM

GC–SIM was performed using a DX303 GC–MS system under the same conditions used for GC–MS with a dynamic resolution of 1000. The base peak ions of $[M-CH_3COOH-OCH_3]^+$ at *m/z* 698.4, *m/z* 684.3, *m/z* 703.4 and *m/z* 687.4 for the TMS derivatives of aconitine, mesaconitine, d_5-aconitine and d_3-mesaconitine, respectively, were monitored.

2.5. Sample preparation of Aconitum alkaloids from human fluid

Extraction and purification of *Aconitum* alkaloids were carried out according to the method reported previously [14] with a small modification. One ml of spiked human fluid was applied in test tube. After adding 1 ng of I.S.s, the sample was deproteinized by adding 10 ml of ethanol. The sample was vortexed and centrifuged at 3000 *g* for 10 min and the supernatant was obtained. After centrifugation, 10 ml of ethanol was poured onto the residue and the same procedure was repeated. The combined supernatant was evaporated until dry and then the residue was dissolved using acetonitrile (0.25 ml, twice). The resulting solution was applied to a Bond Elut Si cartridge and the cartridge was washed with chloroform (10 ml) and then ethyl acetate (10 ml). The alkaloids were eluted with diethylamine–chloroform (1:1, 10 ml). The eluate was evaporated until dry and the residue was silylated with 20 μl of BSTFA in 20 μl of pyridine overnight at room temperature.

3. Results and discussion

I.S.s are commonly used for the quantitative determination of chemical compounds in both biological fluids and tissues by the GC–SIM procedure. Analogues of the compounds that are labelled with three or more deuterium atoms at appropriate positions are considered to be the most effective I.S.s for the applications. Thus, d_5-aconitine and d_3-mesaconitine were synthesized by substitution of the *N*-alkyl group.

Using the procedure mentioned in Section 2, d_5-aconitine and d_3-mesaconitine were synthesized giving a yield of 20% each from aconitine. The ^{1}H NMR signal at δ 1.09 derived from *N*-alkyl protons disappeared (data not shown) owing to the substitution by deuterium atoms. The homogeneity of these compounds were assured by GC and melting points.

Before a specific deuterated analogue was adopted as an I.S., the mass spectrum of this TMS derivative had to be evaluated along with the corresponding spectrum from the TMS derivative of the parent compound. Fig. 2 shows the mass spectra of the TMS derivatives of aconitine and its deuterium-labelled analogue. Aconitine, mesaconitine and their deuterium-labelled analogue were converted into bisTMS ether derivatives, respectively. The fragmentation pattern of their TMS derivatives is summarized in Table 1. As for the TMS derivative of aconitine, the molecular ion was not observed but the

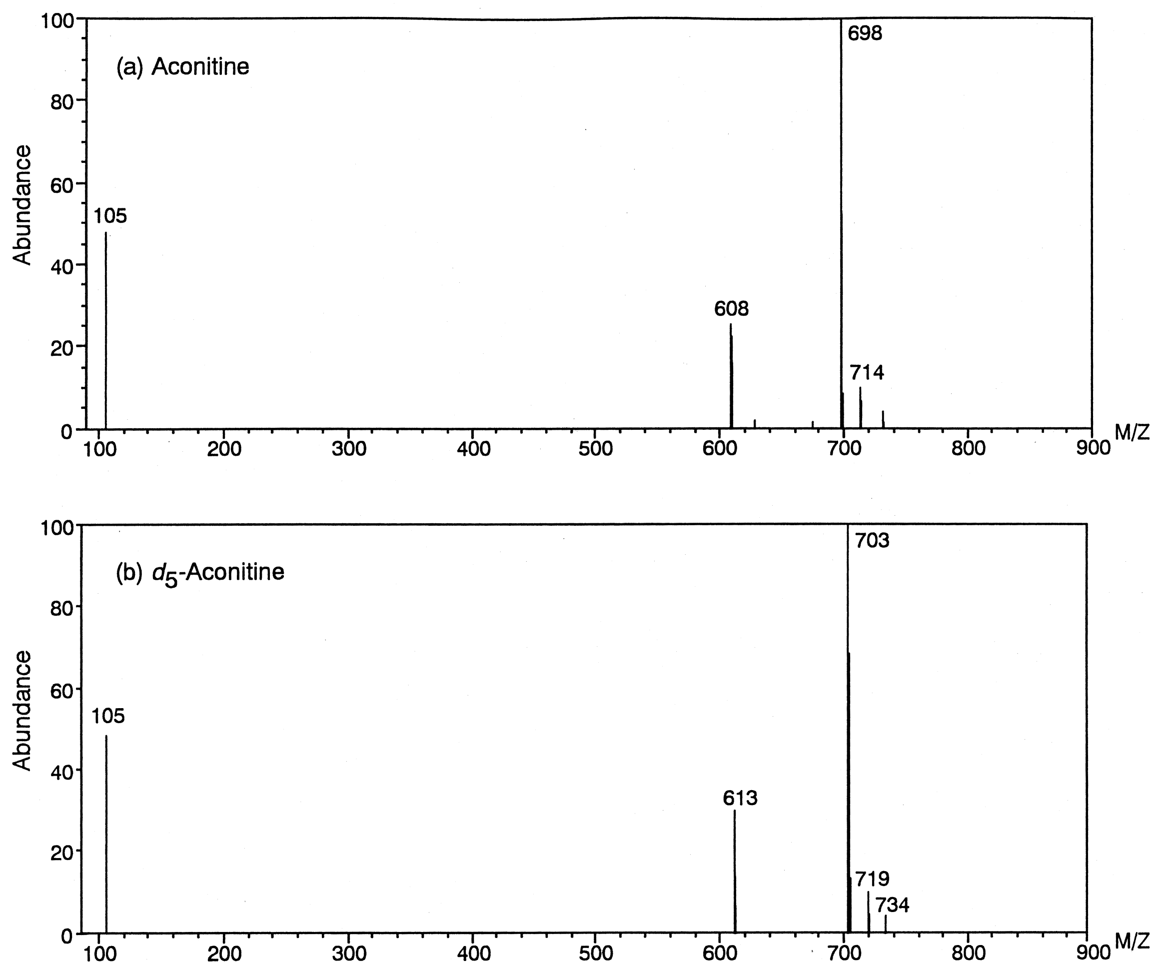

Fig. 2. Mass spectra of the TMS derivatives of aconitine (a) and d_5-aconitine (b).

Table 1
Fragmentations of acetonitine, d_5-aconitine, mesaconitine and d_3-mesaconitine

Fragmentation	bisTMS aconitine	bisTMS d_5-aconitine	bisTMS mesaconitine	bisTMS d_3-mesaconitine
$[M-CH_3COOH]^+$	$C_{38}H_{59}NO_9Si_2$ 729	$C_{38}N_{54}NO_9Si_2D_5$ 734	$C_{37}H_{57}NO_9Si_2$ 715	$C_{37}N_{54}NO_9Si_2D_3$ 718
$[M-CH_3COOH-CH_3]^+$	$C_{37}H_{56}NO_9Si_2$ 714	$C_{37}H_{51}NO_9Si_2D_5$ 719	$C_{36}H_{54}NO_9Si_2$ 700	$C_{36}H_{51}NO_9Si_2D_3$ 703
$[M-CH_3COOH-OCH_3]^+$	$C_{37}H_{56}NO_8Si_2$ 698	$C_{37}H_{51}NO_8Si_2D_5$ 703	$C_{36}H_{54}NO_8Si_2$ 684	$C_{36}H_{51}NO_8Si_2D_3$ 687
$[M-CH_3COOH-OCH_3-TMSOH]^+$	$C_{34}H_{46}NO_7Si$ 608	$C_{34}H_{41}NO_7SiD_5$ 613	$C_{33}H_{44}NO_7Si$ 594	$C_{33}H_{41}NO_7SiD_3$ 597

Each TMS derivative of the alkaloids gave the base peak ions of $[M-CH_3COOH-OCH_3]^+$ at m/z 698, 703, 684 and 687.

ions of $[M-CH_3COOH]^+$ (m/z 729) and $[M-CH_3COOH-CH_3]^+$ (m/z 714) appeared with low intensity. Loss of trimethylsilanol from the base peak of m/z 698 gave the ion of m/z 608, and the ion of m/z 105 was characteristic of the benzoyl ester moiety. The fragmentation patterns of the TMS derivative of mesaconitine was closely related to those of the TMS derivative of aconitine. In the mass spectrum of the deuterium-labelled analogue, the shift of the base peak ion of $[M-CH_3COOH-OCH_3]^+$ from m/z 698 to 703 represents incorporation of five deuterium atoms, and the fragmentation products by simple band fission mechanisms such as the ions of $[M-CH_3COOH_3-CH_3]^+$, $[M-CH_3COOH]^+$ and $[M-CH_3COOH-OCH_3-TMSOH]^+$ were also observed with a shift of five mass units being the same as the base peak ion. In turn, the ion $[C_7H_5O]^+$, derived from the benzoyl moiety at C_{14} (m/z 105), remained as it was. The same result was obtained as with the deuterium-labelled mesaconitine with the difference in the numbers corresponding to the deuterium atoms (Fig. 3).

The intensity ratio of the ions at m/z 698 to 703 for aconitine and ions at m/z 684 to 687 for mesaconitine in the derivatives of the I.S.s were both less than 0.1%, respectively. As for the sensitivity, the detection limit was 10 pg per injection (absolute number) for aconitine, mesaconitine and their I.S.s with a signal to noise (S/N; S/N=peak height/oscillation of noise) ratio greater than 10. Regarding specificity, as the specificity of the SIM methods depend on the number of diagnostic ions, to be sure, one single ion is not very specific. However, the absolute recovery of aconitine and mesaconitine from the blood was 89.0±5.1% and 94.4±1.7% (mean±S.D.) and was obtained using a single ion as their diagnostic ions in a previous paper [14]. In this present study, we used only one diagnostic ion for the alkaloids for simplification.

To examine the suitability of deuterium-labelled analogues as I.S.s, calibration graphs were prepared from GC–SIM analysis of increasing amounts of aconitine and mesaconitine to which was added a constant amount of the I.S. (1 ng). The calibration graph for these alkaloids was obtained by plotting the peak–area ratio of aconitine to an I.S. against their mass ratios. Good linearity was observed in the range 50 pg–50 ng, respectively (r^2=0.999 each).

In order to examine reproducibility, accuracy and precision, three different concentrations of the alkaloids were spiked in the healthy donor's human urine and blood (500 pg, 1 ng, 5 ng for 1 ml of the urine and 100 pg, 500 pg, 1 ng for 1 ml of the blood) and the recovery during the analysis was evaluated. The results are shown in Tables 2 and 3. The mean recovery of these added alkaloids ranged from 97.6 to 101.3%, showing better recoveries and relatively smaller deviations compared to our previous report [14].

In GC–SIM work on extracts from biological materials, it is inevitable that impurities in the sample will exhibit ions with the same nominal masses as those characteristic of the compounds of interest. The typical SIM results from human urine and blood are shown in Fig. 4, which illustrates the SIM traces for four masses corresponding to the base peak ions of aconitine, mesaconitine and their I.S.s. Peaks appearing on the selected ion recording traces corresponded to 100 pg of the alkaloids in 1 ml of

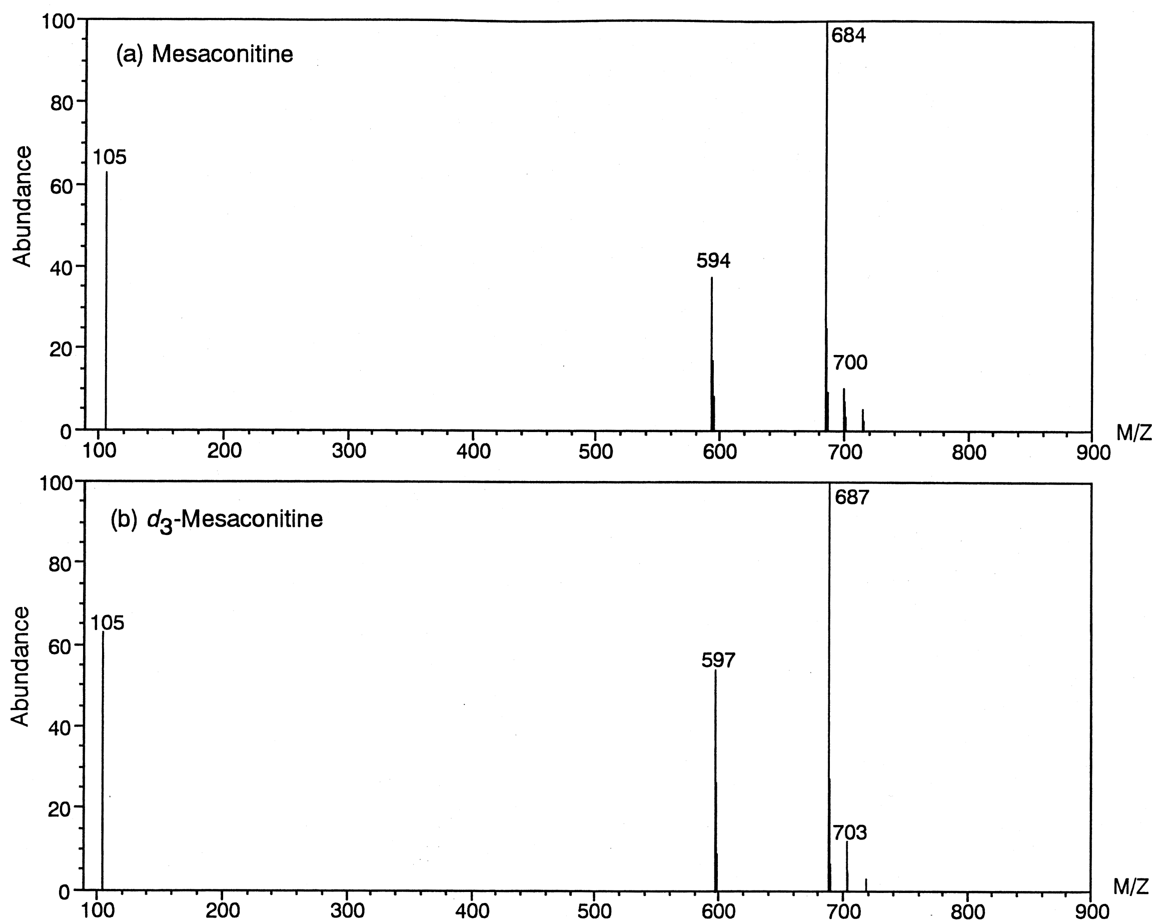

Fig. 3. Mass spectra of the TMS derivatives of mesaconitine (a) and d_3-mesaconitine (b).

the blood and to 500 pg of the alkaloids in 1 ml of the urine each added with 1 ng of d_5-aconitine and d_3-mesaconitine. These figures show that interfering substances from the urine and blood matrix were eliminated during microanalysis and peaks interfering with the quantitation were not observed in the blank urine and blood samples (data not shown). These findings show the I.S.s reported here are very useful for the microdetermination of aconitine and mesaconitine in biological fluids.

As to the metabolism of the alkaloids, we previously reported that one of the metabolites of the

Table 2
Recovery of aconitine and mesaconitine from human urine using their deuterium-labelled analogue as internal standard

Addition (ng/ml)	Recovery (mean±S.D., n=5) (%)	
	Aconitine	Mesaconitine
0.500	100.5±3.4	101.1±2.1
1.000	97.6±3.9	97.9±2.7
5.000	99.0±2.2	100.7±2.4

Table 3
Recovery of aconitine and mesaconitine from human blood using their deuterium-labelled analogue as internal standard

Addition (ng/ml)	Recovery (mean±S.D., n=5) (%)	
	Aconitine	Mesaconitine
0.100	101.3±3.2	100.1±3.5
0.500	97.8±3.1	98.5±3.7
1.000	100.7±2.7	98.7±3.0

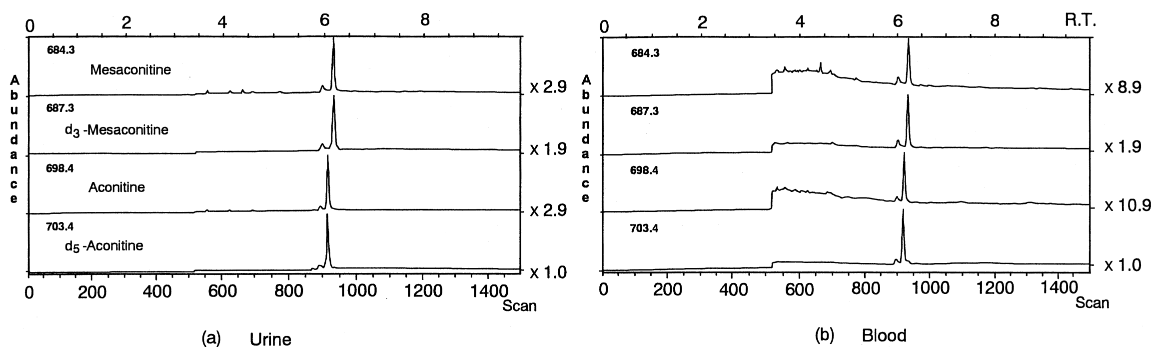

Fig. 4. Selected ion recordings (SIR) of the TMS derivatives of aconitine, d_5-aconitine, mesaconitine and d_3-mesaconitine obtained from human urine (a) and blood (b). The spiked concentration of the alkaloids was 500 pg each for 1 ml of urine and 100 pg each for 1 ml of the blood, added with 1 ng of d_5-aconitine and d_3-mesaconitine.

alkaloids are their hydrolysis products [16]. Although it should be needed for identification, the *N*-desethylaconitine arising during the synthesis of the I.S.s could be possibly metabolite. Therefore, we synthesized deuterium-labelled analogues for simultaneous analysis of diester diterpene type *Aconitum* alkaloids and their metabolites.

There have been some reports on poisoning by aconite where a quantitative analysis of *Aconitum* alkaloids was performed and the concentrations of the alkaloids were reported as in the order of a hundred pg per ml in the serum and ng per ml in the urine [16,17]. Therefore, the I.S.s reported here give us a more precise analysis and are useful for examining the behavior of these alkaloids in human body.

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